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Prostaglandin E_2 is a potent regulator of interleukin-12- and interleukin-18-induced natural killer cell interferon-γ synthesis

WILLIAM WALKER* & DINO ROTONDO† *Experimental Medicine Unit, Swansea Clinical School, University of Wales Swansea, Swansea, UK, and †Department of Immunology, Strathclyde Institute for Biomedical Sciences, University of Strathclyde, Glasgow, UK

SUMMARY

Synthesis of interferon (IFN)-γ by natural killer (NK) cells is an important pro-inflammatory event with interleukin (IL)-12 and IL-18 playing major inductive roles. However, other temporal events are likely to regulate such processes and as prostaglandin E_2 (PGE_2) is ubiquitous during inflammation this study tested the hypothesis that PGE_2 was capable of directly modulating cytokine-induced NK cell IFN-γ synthesis in the absence of other immune cells. Using homogenous NK cell lines to establish direct effects, PGE_2 (0·1–1 μM) was found to suppress NK cell IFN-γ synthesis and antagonized the potent synergistic IFN-γ-inducing effects of IL-12 and IL-18. The actions of PGE_2 were mimicked by synthetic PGE_2 analogues including misoprostol and butaprost. The selective EP_2 receptor agonist butaprost, but not the EP_1/EP_3 agonist sulprostone, suppressed IFN-γ synthesis and exclusively competed with PGE_2 for receptor binding on NK cells. Further analysis showed that PGE_2 did not modulate IL-12 receptor mRNA expression and the effects of PGE_2 could be mimicked by the phosphodiesterase inhibitor 3-iosobutyl-1-methylxanthine. The absence of demonstrable receptor modulation coupled with the observed suppression of IFN-γ synthesis by both EP_2 receptor-selective agonists and IBMX suggest that PGE_2 acts directly on NK cells via EP_2 receptors with its downstream effects on cAMP metabolism. This conclusion is further supported by findings that PGE_2 and its analogues consistently elevated levels of cAMP in NK cells. The ability of PGE_2 to antagonize the potent inductive signal provided by the combination of IL-12 and IL-18 supports the concept that PGE_2 may play an important role in limiting innate inflammatory processes *in vivo* through direct suppression of NK cell IFN-γ synthesis.

INTRODUCTION

Natural killer (NK) cells play a vital role in resistance to cancer and various infectious agents that include viruses, bacteria and intracellular protozoa.[1–3] Upon activation NK cells synthesize interferon-γ (IFN-γ)), a major pro-inflammatory cytokine that is essential for activating macrophage microbicidal activity[4] and also for promoting subsequent T helper 1 (Th1)-like immunity.[5] The induction of NK cell IFN-γ synthesis occurs rapidly, primarily in response to macrophage and dendritic cell-derived interleukin-12 (IL-12)[6] and this process is amplified by other pro-inflammatory cytokines especially IL-18, which acts in synergy with IL-12.[7–10] The importance of these innate events are aptly demonstrated by *in vivo* studies showing that the absence of either IL-12 or IL-18 results in an inability to mount early IFN-γ-mediated resistance and profoundly impaired Th1-like responses to intracellular pathogens.[11]

In addition to regulating numerous physiological processes throughout the body, prostaglandin E_2 (PGE_2) is ubiquitously expressed in many tissues during inflammation and is synthesized by diverse cell types that include immune cells, fibroblasts and skin cells. In the context of innate immunity, NK cell activity involves close interaction with activated dendritic cells and macrophages[12] and significantly, these cell types are major producers of PGE_2.[13] Given the synthesis of PGE_2 by such immune cells, it is perhaps not surprising that PGE_2 has been shown to modulate various aspects of the immune response. For example, PGE_2 has been shown to inhibit lipoloysaccharide (LPS)-induced IL-12 production from

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Correspondence: Dr W. Walker, Experimental Medicine, Swansea Clinical School, University of Wales Swansea, SA2 8PP. E-mail: w.walker@swansea.ac.uk

monocytes[14] decrease IL-2-driven T-cell proliferation[15] and suppress IFN-γ synthesis by $CD4^+$ or $CD8^+$ T cells.[16,17] However, conflicting reports have demonstrated PGE_2-mediated enhancement of IFN-γ synthesis by antigen-stimulated Th1 cells[18] and enhanced dendritic cell IL-12 production in the absence of LPS stimulation.[19] These contradictory findings may be because of differences in cellular activation or on the differential expression of PGE_2 receptors (EP_1–EP_4) as these individual G-protein-coupled receptors utilize distinct intracellular signalling pathways. For example, EP_2/EP_4 receptors act via G_s proteins to increase intracellular cAMP, and the three isoforms of the EP_3 receptor act via G_i proteins to suppress cAMP levels.[20]

In contrast to T lymphocytes, the effects of PGE_2 on NK cell IFN-γ synthesis has received considerably less attention. In particular, given the importance of this fundamental event to host immunity, it was pertinent to determine whether PGE_2 can directly modulate the ability of IL-12-, IL-18-induced NK cell IFN-γ synthesis in the absence of other immune cells. In addressing this important question we demonstrate that physiologically relevant concentrations of PGE_2 significantly suppresses NK cell IFN-γ synthesis, irrespective of cytokine stimulation. Furthermore, this suppression is a result of the direct action of PGE_2 on NK cells, involving EP_2 receptors and the downstream modulation of intracellular cAMP levels. This infers that concomitant production of PGE_2 may be an important innate event that regulates NK cell activity *in vivo*.

MATERIALS AND METHODS

Propagation of NK cells

IL-2-dependent murine NK cell lines (5E3, 5E6) were obtained from Dr Hiroko Tsutsui, Department of Immunology & Medical Zoology, Hyogo College of Medicine, Japan and maintained as described.[21] These cell lines are non-transformed and exhibit identical characteristics to primary NK cells obtained as previously described by us.[10] Briefly, cells were cultured in RPMI-1640 containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 10 mg/ml streptomycin, 1 mM sodium pyruvate, 10 mM HEPES and 1% non-essential amino acids. Cells were seeded at an initial density of 1×10^5 cells/ml and maintained in 500 U/ml of recombinant human IL-2 (Peprotech EC Ltd, London, UK). The IL-2 was replenished every 5–6 days when cells reached confluence. In all experiments NK cells were plated at a final density of 1×10^6/ml in either 24-well tissue culture plates (1 ml/well) or in the case of larger cultures (5 ml) in 25 cm^2 tissue culture flasks.

Stimulation of NK cells

In all stimulation experiments NK cells were cultured with recombinant murine IL-12 and/or IL-18 (Peprotech EC, Ltd) at a final concentration of 1 ng/ml. Tissue culture grade PGE_2 was obtained from Sigma-Aldrich Co. Ltd (Poole, UK) and used at a concentration of 1 μM unless otherwise stated. The PGE_2 analogues butaprost, misoprostol (selective EP_2 agonists) and sulprostone (EP_1/EP_3) were obtained from Cayman Chemical Co. (Ann Arbor, MI) and were used at a final concentration of 10 μM. The cAMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was added to cultures at a final concentration of 100 μM.

Measurement of IFN-γ synthesis

IFN-γ levels in culture supernatants were measured using a standard capture cytokine enzyme-linked immunosorbent assay (ELISA) protocol. Antibody pairs were supplied by BD-PharMingen (Oxford, UK) and used as recommended. Briefly, the wells of Immulon™ microtitre plates (Dynex Technologies Inc., Chantilly, VA) were coated with detecting monoclonal antibody (R4-6A2) in phosphate-buffered saline (PBS) pH 9·0. After addition of samples, bound cytokine was detected with biotinylated anti-IFN-γ (XMG1.2) followed by streptavidin–alkaline phosphatase conjugate. Bound phosphatase was visualized with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (Sigma-Aldrich Chemical Co.). Unknown values were extrapolated from a standard curve which was constructed using recombinant mouse IFN-γ of known concentration.

Ligand-binding analysis

PGE_2 receptor-binding analysis was performed as previously described.[22] Briefly, washed NK cells (1×10^6) were incubated at 4° with 3 nM [^{3}H]-PGE_2 (Amersham Biosciences UK Ltd, Little Chalfont, UK) alone or in combination with 1000-fold molar excess concentrations (3 μM) of either unlabeled PGE_2 or its analogues (butaprost, sulprostone) for 60 min in a total volume of 200 μl. After incubation cells were vacuum-filtered through Whatman GF/C glass-fibre filter papers, presoaked with Tyrode's buffer. Following washing, the filters were dried and radioactivity measured by liquid scintillation counting.

Cytokine receptor expression

To detect cytokine receptor expression total RNA was extracted from cultured NK cells by the guanidine isothiocyanate method and assayed for cytokine receptor mRNA content using the Riboquant™ Multi-Probe RNase Protection Assay System (BD-PharMingen). Briefly, 10 μg of RNA from each sample was hybridized in solution with the appropriate radiolabelled antisense RNA probe set (mCR-1, mCR-3) as recommended by the manufacturers. Following hybridization, free probe and remaining single stranded RNA was digested with RNases and the protected probes were purified and resolved on 5% denaturing polyacrylamide gels using Ultra Pure Sequagel™ reagents (National Diagnostics, Atlanta, GA). Protected fragments were visualized by exposing dried gels to standard X-ray imaging film. Post-development films were scanned and individual band densities analysed by NIH-*Image* computer software.

Measurement of intracellular cAMP levels

NK cells (1×10^6) were cultured in the presence of PGE_2 or its synthetic analogues in a final volume of 250 μl RPMI-1640. Reactions were terminated after 30 min by the addition of trichloroacetic acid (TCA, 5% final concentration). Supernatants were then collected into fresh tubes and TCA removed by washing three times with 4 volumes of diethyl ether. cAMP was subsequently measured in samples by radioimmunoassay. Briefly, a constant amount of radiolabelled cyclic AMP and mouse anti-cAMP antibody was added to each sample. Samples were then incubated at 4° for 18 hr after which a second

antibody (goat anti-mouse), conjugated to magnetic beads, was added followed by ice-cold polyethylene glycol 6000 (12% final concentration). Samples were then vortex-mixed and centrifuged at 13 000 ***g*** to remove the supernatant. Radioactivity in the pellet was measured by scintillation counting and the level of cAMP evaluated by comparison to the radioactivity in tubes containing standard amounts of cAMP.

Statistical analysis

All data are expressed as mean values ± 1 standard deviation (SD). Protein IFN-γ levels were compared by the two-tailed Student's *t*-test. Specific mRNA expression was calculated according to the formula: densitometric values for cytokine receptor bands/L32 band density. Normalized densitometric values under different stimulatory conditions were compared by Mann–Whitney *U*-test. *P*-values less than 0·05 were considered significant.

RESULTS

PGE_2 suppresses IL-12 & IL-18-induced NK cell IFN-γ synthesis

As PGE_2 is known to have modulatory effects on accessory cell functions that are pertinent to NK cell IFN-γ synthesis, i.e. IL-12 production,[14,19] it was important to determine direct effects on NK cells in the absence of these cell types. As contaminating accessory cells are unavoidable during generation of primary NK cell cultures from bone marrow precursors, homogeneous non-transformed NK cell lines were employed.[21] In our hands these NK cells exhibited identical characteristics to primary NK cells generated by other methods.[10] To mimic physiological conditions as much as possible, NK cells were stimulated with only 1 ng/ml final concentrations of cytokine, and up to 1 μM final concentrations of PGE_2, a quantity previously shown to maximally inhibit macrophage IL-12 production.[14] In agreement with our previous findings using primary NK cells[10] IL-12 and IL-18 acted in synergy to significantly enhance production of IFN-γ when compared with stimulation by IL-12 or IL-18 alone (Fig. 1a). However, irrespective of the inducing cytokine stimulus, PGE_2 suppressed the synthesis of IFN-γ. PGE_2 was effective at concentrations as low as 0·01 μM with a maximal suppressive effect between 0·1 and 1 μM (Fig. 1b). Despite the potent synergism of IL-12 and IL-18, 1 μM PGE_2 was able to significantly reduce IFN-γ levels under these conditions (41·5% suppression). Under weaker stimulatory conditions the suppressive effects of PGE_2 were more profound with IL-12- and IL-18-stimulated IFN-γ levels reduced on average by 58 and 67%, respectively, suggesting a direct antagonism between IFN-γ-inducing stimuli and PGE_2. These inhibitory effects were not caused by cell death as PGE_2 did not have any significant effect on cell viability during the relatively short culture conditions (16 hr) as determined by trypan blue exclusion (not shown). We did not perform experiments with excess PGE_2 (i.e. >1·0 μM) as these levels are unlikely to reflect pathophysiological conditions given the very short half-life of PGE_2 *in vivo*.[23,24] These findings show that cytokine-induced NK cell IFN-γ synthesis is profoundly regulated in a negative manner by PGE_2.

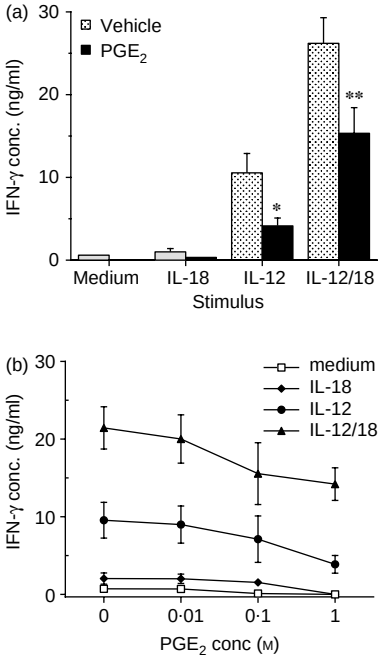

Figure 1. PGE_2 suppresses IL-12- and IL-18-induced IFN-γ synthesis by NK cells. (a) NK cells (1×10^6) were stimulated with IL-12 ± IL-18 (1 ng/ml) for 16 hr in the absence (ethanol vehicle) or presence of 1·0 μM PGE_2 and IFN-γ in culture supernatants measured by standard capture ELISA. The synergistic combination of IL-12 + IL-18 in these experiments was susceptible to the suppressive effects of PGE_2 with an average reduction in IFN-γ levels of 41·5%, $^{**}P < 0{\cdot}05$ (μ = 26·2 ± 3·1 ng/ml to 15·33 ± 3·0 ng/ml). IL-12-stimulated IFN-γ levels were reduced on average by 58·6%, $^{*}P < 0{\cdot}05$ (μ = 10·55 ± 2·3 ng/ml to 4·1 ± 0·9 ng/ml). IL-18-induced IFN-γ levels (1·1 ± 0·4 ng/ml) were more markedly reduced with a 67% reduction. Measurable background IFN-γ levels (μ = 0·6 ng/ml) were completely abolished in the presence of 1·0 μM PGE_2. $n = 3 \pm$ SD. (b). Dose–response experiments further confirmed that PGE_2 suppressed the production of IFN-γ under all stimulatory conditions with PGE_2 having a maximal suppressive effect between 0·1 and 1·0 μM final concentration. Data is representative of 3 individual experiments, error bars = ± SD. The IC_{50} for PGE_2 in the presence of IL-12/18 = 360 nM. In the presence of IL-12 or IL-18 the IC_{50} for PGE_2 = 33 nM and 20 nM, respectively.

PGE_2 mediates its effects on NK cell IFN-γ synthesis via EP_2 receptors

Given the marked suppression of IFN-γ synthesis in the absence of accessory cells it was important to determine the molecular basis for these direct effects. To determine which PGE_2 (EP) receptors where involved we employed synthetic analogues of PGE_2 that act as selective EP receptor agonists. When NK cells were stimulated in the presence of the selective EP_2 receptor agonist butaprost or the $EP_2/EP_3/EP_4$-active analogue misoprostol, a significant inhibition of IFN-γ synthesis occurred in a manner analogous to that seen with natural PGE_2 (Fig. 2). Butaprost suppressed IL-12 + IL-18-induced IFN-γ levels on average by 33% (from 26·3 ng/ml to 17·65 ng/ml) with misoprostol also exhibiting similar effects (reduced to 15·4 ng/ml) when compared with natural PGE_2 (15·3 ± 5·1 ng/ml). Similar to PGE_2, butaprost and misoprostol also suppressed more

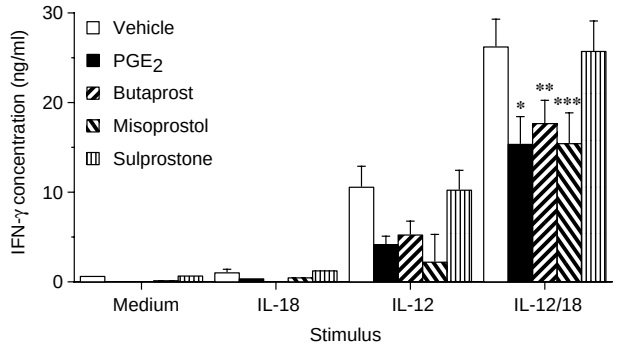

Figure 2. PGE_2 analogues selective for EP_2 receptors mediate suppressive effects on NK cell IFN-γ synthesis. NK cells (1×10^6) were stimulated with cytokines for 16 hr in the presence of either PGE_2 (1 μM), butaprost (EP_2 agonist, 10 μM), misoprostol ($EP_2/EP_3/EP_4$ agonist, 10 μM) or sulprostone (EP_1/EP_3 agonist, 10 μM) and IFN-γ production measured by ELISA. In the presence of IL-12 + IL-18 butaprost reduced mean IFN-γ levels from 26·3 ± 4·1 ng/ml to 17·65 ± 2·6 ng/ml (33% reduction, $^{**}P < 0{\cdot}05$, $n = 3 \pm$ SD) compared with a similar reduction in the presence of PGE_2 (to 15·3 ± 5·1 ng/ml, $^{*}P < 0{\cdot}05$). Similarly, misoprostol also inhibited IL-12 + IL-18-induced IFN-γ synthesis by NK cells (mean IFN-γ synthesis in the presence of misoprostol = 15·4 ± 3·4 ng/ml, $^{***}P < 0{\cdot}05$). Sulprostone had no demonstrable effect on IFN-γ synthesis (mean IFN-γ concentration = 25·7 + 3·4 ng/ml in the presence of sulprostone, $P > 0{\cdot}05$ compared with vehicle). The absence of EP_1/EP_3-mediated effects (sulprostone) combined with the activity of misoprostol and butaprost, strongly indicate the involvement of EP_2 receptors in IFN-γ suppression. As shown, similar effects were discernable when these analogues were added to NK cells stimulated in the presence of IL-12 or IL-18 only. $n = 3 \pm$ SD.

efficiently the synthesis of IL-12-stimulated or IL-18-stimulated IFN-γ production. In contrast, the selective EP_1/EP_3 receptor agonist sulprostone did not have any significant effect on IL-12-or IL-18-induced IFN-γ synthesis (μ = 25·7 ng/ml), suggesting an absence of EP_1/EP_3 receptors on activated NK cells.

Although butaprost has been well characterized as a selective agonist for the EP_2 receptor, it was desirable to rule out possible indirect effects of this drug on NK cells and also to confirm that the EP_2 receptor was the major ligand for PGE_2 on the surface of NK cells. Therefore, radio-ligand binding analysis was performed in which PGE_2 and its analogues were allowed to compete with $[^3H]$-PGE_2 for binding to NK cells (Fig. 3). Total binding of PGE_2 was significantly reduced only by the presence of either unlabelled PGE_2 or the EP_2-selective agonist butaprost (reduced to 53·4% ± 7·3% and 48·6% ± 5·5%, respectively, $P < 0{\cdot}05$). In contrast, sulprostone did not have any significant effect on radiolabelled PGE_2 binding, confirming the absence of significant numbers of EP_1/EP_3 receptors on NK cells. Given that butaprost does not interact with the EP_1, EP_3, or EP_4 receptors[25–27] these findings strongly support the conclusion that PGE_2 mediates its effects on NK cells predominantly through the EP_2 receptor.

PGE_2 does not modulate IL-12 cytokine receptor expression on NK cells

Previous studies with T lymphocytes have indicated that PGE_2 has the ability to down-regulate the IL-12 receptor β subunits

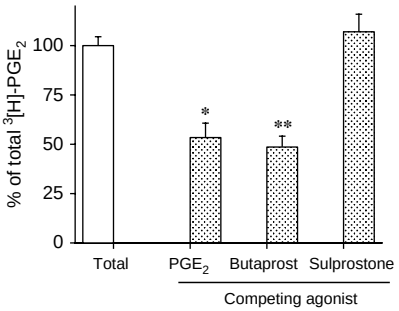

Figure 3. NK cells predominantly express EP_2 receptors. Washed NK cells (1×10^6) were incubated with 3 nanomolar $[^3H]$-PGE_2 in the presence of excess (3 μM) synthetic EP receptor agonists and bound $[^3H]$-PGE_2 determined by liquid scintillation counting. In the presence of unlabelled PGE_2 binding was reduced to 53·4 ± 7·3% ($^{*}P < 0{\cdot}05$). In the presence of butaprost binding was reduced to 48·6 ± 5·5% ($^{**}P < 0{\cdot}05$) demonstrating EP_2 receptor expression. In contrast, coincubation with sulprostone did not result in any alteration in $[^3H]$-PGE_2 binding (107 ± 8·9%, $P > 0{\cdot}05$) confirming the absence of EP_1 or EP_3 receptors on NK cells. Total binding was 290 ± 21 dpm/10^6 cells ($n = 4$, ± SD).

(IL-12Rβ)[28] and as such a mechanism could account for the observed suppression, the ability of PGE_2 to modulate NK cell IL-12R expression was therefore determined. Although not involved in either IL-12 or IL-18 signaling, the effects of PGE_2 on expression of the IL-2R subunits (α, β, γ_c) were also evaluated as an indicator of any nonspecific effects. As an additional control, the effects of PGE_2 were compared with TGF-β1, a reported inhibitor of IL-12Rβ2 expression on T cells.[29] As previous studies have demonstrated a direct correlation between IL-12R mRNA and cell-surface protein expression[30] we measured IL-12Rβ mRNA subunit expression by RNase protection assay (RPA). Irrespective of the stimulatory conditions, PGE_2 treatment did not inhibit expression of mRNA for either the IL-12 or IL-2 receptor subunits (Fig. 4). In comparison, down-regulation of both IL-12R subunits at the mRNA level was detectable after TGF-β1 treatment for 16 hr (not shown), validating the sensitivity of the mRNA assay. Therefore, PGE_2 does not suppress cytokine-induced IFN-γ synthesis by a mechanism that involves down-modulation of IL-12 receptors on NK cells. Furthermore, the absence of demonstrable down-regulation of other cytokine receptors (including the IL-2R family subunits), suggests that at least under the short treatment time used here, PGE_2 does not significantly modulate cytokine receptor expression on NK cells.

IFN-γ synthesis by NK cells is suppressed in a cAMP-dependent manner

As PGE_2 did not modulate expression of IL-12 receptors, more direct effects on IFN-γ gene expression could account for its suppressive effects. In particular, EP_2 receptors signal via G_s proteins to increase intracellular cAMP levels[20] and it was therefore pertinent to test whether PGE_2 was capable of modulating cAMP levels in NK cells. To achieve this, cAMP levels in NK cells were determined by radioimmunoassay after

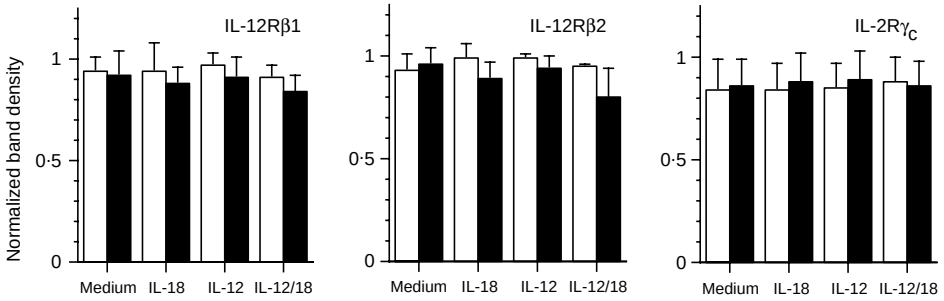

Figure 4. PGE_2 does not modulate NK cell cytokine receptor expression. NK cells (5×10^6) were stimulated with IL-12 ± IL-18 (1 ng/ml) for 16 hr in the absence (ethanol vehicle) or presence of PGE_2 (1·0 μM) and cytokine receptor mRNA levels determined by RPA. Irrespective of the IFN-γ-inducing conditions, PGE_2 did not modify IL-12R β1/β2 chain mRNA expression (left and middle panels). Similarly, PGE_2 exposure for 16 hr did not modulate IL-2R subunit (γ_c) mRNA expression (right panel). IL-2R α or β chain mRNA expression was also unaffected by PGE_2 exposure (not shown). Shown is the mean normalized expression (represented as the ratio of receptor band density/L32 band density) + SD, $n = 3$. White bars = vehicle control, black bars = PGE_2-treated cultures.

treatment with PGE_2 or its synthetic analogues. In the presence of either PGE_2, misoprostol or butaprost, cAMP levels were significantly elevated compared with untreated cells (Fig. 5a). This effect was most marked at the 10 μM and 1·0 μM concentrations. Lower concentrations (0·1 μM) failed to elevate cAMP levels above background control levels (654 ± 139 fmol). In contrast, treating NK cells with identical concentrations of sulprostone did not produce any significant elevation in intracellular cAMP levels. In further experiments, NK cells were incubated with equivalent amounts (1·0 μM) of either PGE_2 or butaprost plus sulprostone. Despite the presence of sulprostone in these cultures, PGE_2 and butaprost were still capable of elevating NK cell cAMP levels (Fig. 5b). As sulprostone preferentially utilizes PGE_2 receptors that are G_i-coupled (EP_3) and inhibitory of cAMP generation, any sulprostone binding would predict an antagonism of the cAMP-elevating effects of butaprost or PGE_2. As this was not observed, these experiments provide further evidence of the absence of EP_3-mediated effects on NK cells. Finally, to directly test whether cAMP-dependent mechanisms could account for the suppression of IFN-γ synthesis, intracellular cAMP levels were artificially elevated in cytokine-stimulated NK cells by coincubating with the cAMP-phosphodiesterase inhibitor IBMX. As observed with natural PGE_2, IBMX suppressed the synthesis of IFN-γ under all stimulatory conditions (Fig. 5c). Similar to PGE_2, IBMX was also more efficient at suppressing IL-12-stimulated IFN-γ synthesis (64% reduction) than the synergistic effects of IL-12 plus IL-18 (30% reduction). IL-18-induced and background levels of IFN-γ were abolished in the presence of IBMX. PGE_2 plus IBMX combined had an additive effect with a more marked suppression of IFN-γ synthesis than either of these agents on their own, although this was not significant in the IL-12 plus IL-18-stimulated cultures. These findings demonstrate that IFN-γ expression is suppressed via a cAMP-dependent mechanism and this is consistent with the data from selective agonists (Fig. 2) and radio-ligand binding analysis (Fig. 3) showing that PGE_2 acts via EP_2 receptors on NK cells. They also support our observations that indirect effects such as cytokine-receptor modulation (Fig. 4) are not required for suppression of NK cell IFN-γ synthesis.

DISCUSSION

Because prostanoids induce the symptomatic manifestations of tissue inflammation they were initially characterized as pro-inflammatory mediators. However, it has become clear that they can both promote inflammation and regulate the actions of cytokines which initiate the inflammatory cascade. This depends on many factors such as the inflammatory stimulus, prostanoid type, and receptor expression, leading to the concept that these lipid molecules are both effectors and regulators of inflammation.[31] In agreement with this concept, we report here that PGE_2 acts directly on NK cells to inhibit cytokine-induced IFN-γ synthesis. During early immune activation NK cell IFN-γ synthesis is driven principally by IL-12 and IL-18[10] with activated macrophages and dendritic cells acting as major producers of these pro-inflammatory cytokines. However, these cell types are also major producers of PGE_2, suggesting coordinated, temporal regulation of NK cell activity. The suppressive effect on NK cell IFN-γ synthesis demonstrated in the present study is in addition to the reported inhibitory effects of PGE_2 on IL-12 synthesis by activated macrophages[14] reflecting an additional layer of regulation. However, unlike macrophages where PGE_2 has also been shown to induce suppression through indirect mechanisms that include the induction of regulatory IL-10[32] the effects on NK cells appear to be direct, in that the use of pure NK cell lines allowed us to analyse effects in the absence of contaminating accessory cells. Although NK cells have been reported to secrete IL-10, this cytokine was undetectable under the pro-inflammatory conditions used in this and other studies[10] and was not detectable after PGE_2 treatment. Furthermore, IL-10 has been shown to have stimulatory effects on NK cell IFN-γ synthesis in the presence of IL-18[33] and such a scenario is therefore an unlikely explanation for the observed suppression.

PGE_2 has been reported to inhibit T cell IFN-γ production[17,34] shifting the balance towards Th2 development[35] and our findings are consistent with this, in that NK cell-derived IFN-γ production is recognized to be important for the promotion of Th1 development.[5] Indeed, it has recently been argued that the higher expression of PGE_2 receptors and therefore enhanced sensitivity to IFN-γ-suppressing effects, account

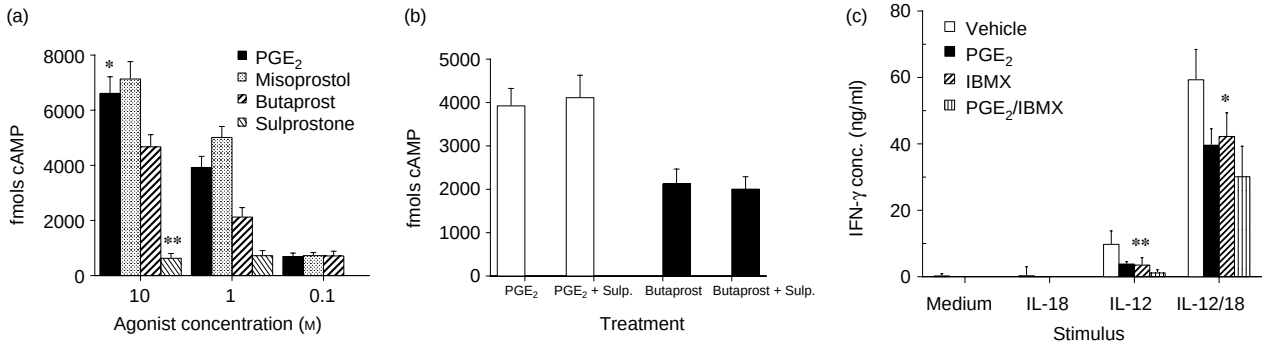

Figure 5. IFN-γ synthesis by NK cells is suppressed by in a cAMP-dependent manner. (a) NK cells (1×10^6) were incubated with PGE_2 or its analogues and cAMP levels determined by radioimmunoassay. In the presence of 10·0 μM or 1·0 μM PGE_2, butaprost or misoprostol cAMP levels were significantly elevated compared with untreated cells (e.g. 10 μM $PGE_2 = 6612 \pm 602$ fmol cAMP vs. 654 ± 139 fmol for untreated cells, $*P < 0{\cdot}005$). In contrast, sulprostone had no effect on intracellular cAMP levels compared with vehicle-treated control cells (10 μM sulprostone $= 627 \pm 174$ fmol, $**P > 0{\cdot}05$). (b) When sulprostone (1·0 μM) was added simultaneously with either PGE_2 (1·0 μM) or butaprost (1·0 μM) there was no significant change in the ability of PGE_2 or butaprost to elevate cAMP levels ($PGE_2 = 3923 \pm 402$ fmol cAMP versus PGE_2 plus sulprostone $= 4112 \pm 517$ fmol, $P > 0{\cdot}05$; butaprost $= 2123 \pm 343$ versus butaprost plus sulprostone $= 2003 \pm 287$), further confirming the absence of EP_1/EP_3-mediated effects. (c) In further experiments, NK cells (1×10^6) were stimulated with IL-12 ± IL-18 (1 ng/ml) for 16 hr in the absence (ethanol vehicle) or presence of either PGE_2 (1 μM) or IBMX (100 μM) to raise intracellular cAMP levels. Similar to PGE_2, IBMX suppressed IFN-γ synthesis irrespective of inducing stimuli. Under stimulation with IL-12 + IL-18, IBMX reduced IFN-γ levels from $59{\cdot}3 \pm 9{\cdot}1$ ng/ml to $42{\cdot}2 \pm 7{\cdot}1$ ng/ml (30% reduction, $*P < 0{\cdot}05$). Under stimulation with IL-12 alone, IBMX reduced IFN-γ levels from $9{\cdot}7 \pm 4{\cdot}0$ ng/ml to $3{\cdot}5 \pm 2{\cdot}1$ ng/ml (64% reduction, $**P < 0{\cdot}05$). When PGE_2 and IBMX were added together there was a small additive effect in terms of suppression, although this was not significant in the IL-12 plus IL-18-stimulated cultures. For all experiments, $n = 3 \pm$ SD.

for the dominant Th2 response in BALB/c mice.[36] Interestingly, EP_2 receptors have been demonstrated to mediate the effects of PGE_2 on both T cells[16] and B cells[37] with more recent studies utilizing EP receptor-deficient mice confirming a major role for the EP_2 receptor in the modulation of T-cell activity.[38] Our findings demonstrate that PGE_2 also utilizes EP_2 receptors on NK cells to regulate IFN-γ production.

Findings with PGE_2 analogues and cAMP-elevating agents such as IBMX present a compelling case for cAMP-mediated regulation of NK cell IFN-γ synthesis. Furthermore, given that NK cells were completely insensitive to sulprostone (EP_1/EP_3-selective) and given that $EP_1/EP_3/EP_4$ receptors are insensitive to butaprost[25–27] the conclusion that similar to T cells, PGE_2 mediates effects on NK cells through the EP_2 receptor is valid. However, with regard to the actual molecular basis for these effects we found important differences. Unlike previous findings with anti-CD3-stimulated human mononuclear cells[28] we could not demonstrate any effect of PGE_2 on NK cell IL-12 receptor subunit expression at the mRNA level. Although we did not directly measure IL-12 binding, previous studies have demonstrated a direct and strict correlation between IL-12R mRNA and cell-surface protein expression[30] with PGE_2 shown to have equivalent suppressive effects on both IL-12R protein and IL-12R mRNA expression[28] validating our approach. The insensitivity of NK cell IL-12R expression to PGE_2 contrasts with findings in T cells[28] and emphasizes the differential regulation of the IL-12R between these cell types. For example, NK cells constitutively express the IL-12R and although this expression can be up-regulated by cytokines such as IL-2[39] NK cell IL-12R expression is generally resistant to cytokines that suppress IL-12R expression on T cells.[40] Our findings with regard to IL-2R expression are interesting in that recent experiments with IL-15-stimulated human NK cells showed that PGE_2 treatment for 2 days led to inhibition of the common-γ chain subunit of the IL-2R (γ_c).[41] In contrast, in our studies where PGE_2 exposure was significantly shorter (16 hr maximum) and in the absence of exogenous IL-15, we did not find any significant effect of PGE_2 on expression of IL-2R mRNA transcripts. In any case, IL-12 or IL-18 receptor signalling does not involve the γ_c-chain and would therefore not account for the observed inhibition of IFN-γ synthesis.

The absence of cytokine receptor modulation coupled with strong evidence that PGE_2 utilizes EP_2-, cAMP-dependent mechanisms suggests regulation of intracellular signalling pathways that impinge on IFN-γ gene expression. In the context of cytokine-induced NK cell IFN-γ synthesis IL-12-activated Jak2/STAT4 and IL-18-activated nuclear factor (NF)-κB/AP-1 are particularly relevant as both are required for maximal IFN-γ promoter activity.[42] Indeed, PGE_2 has been shown to inhibit NF-AT, AP-1,[15] NF-κB[43] and Jak signalling[44],although whether this applies to NK cells has not been determined. Interestingly, PGE_2-induced cAMP has also been shown to result in phosphorylation of cAMP-response element binding protein (CREB)[45] and CREB has been heavily implicated in the negative regulation of IFN-γ gene expression through competitive binding to regulatory elements of the IFN-γ promoter.[46,47] Whether PGE_2 modulates IFN-γ gene transcription in NK cells via CREB-induced suppression of pertinent transcription factors has not been determined and we are currently investigating these and other possibilities.

In summary, this study demonstrates an important role for PGE_2 in the regulation of pro-inflammatory cytokine (IL-12 and

IL-18)-induced production of IFN-γ by NK cells. Importantly, PGE_2 was found to act directly and rapidly antagonize the synergistic effects of IL-12 and IL-18 without modulating cytokine receptor expression. In addition, through the use of synthetic PGE_2 analogues and cAMP measurements we conclude that the suppressive effects of PGE_2 are mediated via EP_2 receptors and its downstream effects on cAMP metabolism. These findings demonstrate direct regulatory effects on NK cells and provide additional important information on the regulation of pro-inflammatory processes to those previously described for accessory cells such as macrophages and dendritic cells. We speculate that the direct effect of PGE_2 on NK cells may be an important regulatory event *in vivo*, acting with other regulators to prevent the pathological consequences of IFN-γ overproduction during early immune activation.

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REFERENCES

1 Bancroft GJ. The role of natural killer cells in innate resistance to infection. Curr Opin Immunol 1993; **5:**503–10.
2 Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol 1999; **17:**189–220.
3 Scharton-Kersten TM, Sher A. Role of natural killer cells in innate resistance to protozoan infections. Curr Opin Immunol 1997; **9:**44–51.
4 Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. Annu Rev Immunol 1997; **15:**749–95.
5 Scott P, Trinchieri G. The role of natural killer cells in host–parasite interactions. Curr Opin Immunol 1995; **7:**34–40.
6 Trinchieri G. Immunobiology of interleukin-12. Immunol Res 1998; **17:**269–78.
7 Hunter CA, Timans J, Pisacane P *et al.* Comparison of the effects of interleukin-1α, interleukin-1β and interferon-γ -inducing factor on the production of interferon-γ by natural killer cells. Eur J Immunol 1997; **27:**2787–92.
8 Lauwerys BR, Renauld J-C, Houssiau FA. Synergistic proliferation and activation of natural killer cells by interleukin-12 and interleukin-18. Cytokine 1999; **11:**822–30.
9 Tomura M, Zhou X-Y, Maruo S *et al.* A critical role for IL-18 in the proliferation and activation of $NK1.1^+CD3^-$ cells. J Immunol 1998; **160:**4738–46.
10 Walker W, Aste-Amezaga M, Kastelein RA, Trinchieri G, Hunter CA. IL-18 and CD28 use distinct molecular mechanisms to enhance NK cell production of IL-12-induced IFN-γ. J Immunol 1999; **162:**5894–901.
11 Takeda K, Tsutsui H, Yoshimoto T *et al.* Defective NK cell activity and Th1 response in IL-18 deficient mice. Immunity 1998; **8:**383–90.
12 Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G. Reciprocal activating interaction between natural killer cells and dendritic cells. J Exp Med 2002; **195:**327–33.
13 Phipps RP, Stein RH, Roper RL. A new view of prostaglandin E regulation of the immune response. Immunol Today 1991; **12:**349–52.
14 van der Pouw Kraan TC, Boeije LC, Smeenk RJ, Wijdenes J, Aarden LA. Prostaglandin E_2 is a potent inhibitor of human interleukin-12 production. J Exp Med 1995; **181:**775–9.
15 Paliogianni F, Boumpas DT. Prostaglandin E_2 inhibits the nuclear transcription of the human interleukin 2, but not the IL-4 gene in human T cells by targeting the transcription factors AP-1 and NF-AT. Cellular Immunol 1996; **171:**95–101.
16 Ganapathy V, Gurlo T, Jarstadmarken HO, von Grafenstein H. Regulation of TCR-induced IFN-γ release from islet-reactive non-obese diabetic $CD8^+$ T cells by prostaglandin E_2 receptor signaling. Int Immunol 2000; **12:**851–60.
17 Hilkens CM, Snijders A, Vermulen H, van der Meide PH, Wierenga EA, Kapsenberg ML. Accessory cell-derived IL-12 and prostaglandin E_2 determine the IFN-γ level of activated human $CD4^+$ T cells. J Immunol 1996; **156:**1722–7.
18 Bloom D, Jabrane-Ferrat N, Zeng L *et al.* Prostaglandin E_2 enhancement of interferon-γ production by antigen-stimulated type 1 helper T cells. Cell Immunol 1999; **194:**21–7.
19 Rieser C, Bock G, Klocker H, Bartsch G, Thurnher M. Prostaglandin E_2 and tumor necrosis factor-α cooperate to activate human dendritic cells. synergistic activation of interleukin-12 production. J Exp Med 1997; **186:**1603–8.
20 Sugimoto Y, Narumiya S, Ichikawa A. Distribution and function of prostanoid receptors: studies from knockout mice. Prog Lipid Res 2000; **39:**289–314.
21 Tsutsui H, Nakanishi K, Matsui K, Higashino K, Okamura H, Miyazawa Y, Kaneda K. IFN-γ-inducing factor up-regulates Fas ligand-mediated cytotoxic activity of murine natural killer cell clones. J Immunol 1996; **157:**3967–73.
22 Loh CL, Rotondo D, Dutta-Roy AK. Characterization of prostaglandin E_2 receptors expressed on human monocytic leukaemia cell line, U937. Biochim Biophys Acta 1993; **1177:**43–8.
23 Davidson J, Abul HT, Milton AS, Rotondo D. Cytokines and cytokine inducers stimulate prostaglandin E_2 entry into the brain. Eur J Physiol 2001; **442:**526–33.
24 Samuelsson B, Granstrom E, Green K, Hamberg M, Hammerstrom S. Prostaglandins. Annu Rev Biochem 1975; **44:**669–95.
25 Takahashi S, Takeuchi K, Okabe S. EP_4 receptor mediation of prostaglandin E_2-stimulated mucus secretion by rabbit gastric epithelial cells. Biochem Pharmacol 1999; **58:**1997–2002.
26 Arakawa T, Laneuville O, Miller CA, Lakkides KM, Wingerd BA, DeWitt DL, Smith WL. Prostanoid receptors of murine NIH 3T3 and RAW 264.7 cells. J Biol Chem 1996; **271:**29569–75.
27 Nishigaki N, Negishi M, Sugimoto Y, Namba T, Narumiya S, Ichikawa A. Identification of prostaglandin E receptor 'EP_2' cloned from mastocytoma cells as EP_4 subtype. FEBS Lett 1995; **364:**339–41.
28 Wu C-Y, Wang K, McDyer JF, Seder RA. Prostaglandin E_2 and dexamethasone inhibit IL-12 receptor expression and IL-12 responsiveness. J Immunol 1998; **161:**2723–30.
29 Gorham JD, Guler ML, Fenoglio D, Gubler U, Murphy KM. Low dose TGF-β attenuates IL-12 responsiveness in murine Th cells. J Immunol 1998; **161:**1664–70.
30 Rogge L, Barberis-Maino L, Biffi M, Passini N, Presky D, Gubler U, Sinigaglia F. Selective expression of an interleukin-12 receptor component by human T helper 1 cells. J Exp Med 1997; **185:**817–31.
31 Tilley SL, Coffman TM, Koller BH. Mixed messages. modulation of inflammation and immune responses by prostaglandins and thromboxanes. J Clin Invest 2001; **108:**15–23.
32 Strassmann G, Patil-Koota V, Finkelman F, Fong M, Kambayashi T. Evidence for the involvement of interleukin 10 in differential deactivation of murine peritoneal macrophages by prostaglandin E_2. J Exp Med 1994; **180:**2365–70.
33 Cai G, Kastelein RA, Hunter CA. IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-γ when combined with IL-18. Eur J Immunol 1999; **29:**2658–65.
34 Katamura K, Sintaku N, Yamauchi Y, Fukui T, Ohshima Y, Mayumi M, Furusho K. Prostaglandin E_2 at priming of naive $CD4^+$ T cells

inhibits acquisition of ability to produce IFN-γ and IL-2, but not IL-4 and IL-5. J Immunol 1995; **155:**4604–12.

35 Kaur K, Harris SG, Padilla J, Graf BA, Phipps RP. ProstaglandinE_2 as a modulator of lymphocyte-mediated inflammatory and humoral responses. Adv Exp Med Biol 1999; **469:**409–11.

36 Kuroda E, Sugiura T, Zeki K, Yoshida Y, Yamashita U. Sensitivity difference to the suppressive effect of prostaglandin E_2 among mouse strains: a possible mechanism to polarize Th2 type response in BALB/c mice. J Immunol 2000; **164:**2386–95.

37 Fedyk ER, Phipps RP. Prostaglandin E_2 receptors of the EP_2 and EP_4 subtypes regulate activation and differentiation of mouse B lymphocytes to IgE-secreting cells. Proc Natl Acad Sci USA 1996; **93:**10978–83.

38 Nataraj C, Thomas DW, Tilley SL, Nyguen M, Mannon R, Koller BH, Coffman TM. Receptors for prostaglandin E_2 that regulate cellular immune responses in the mouse. J Clin Invest 2001; **108:**1229–35.

39 Chakir H, Camilucci AA, Filion LG, Webb JR. Differentiation of murine NK cells into distinct subsets based on variable expression of the IL-12Rβ2 subunit. J Immunol 2000; **165:**4985–93.

40 Wu C-Y, Gadina M, Wang K, O'Shea J, Seder RA. Cytokine regulation of IL-12 receptor β2 expression: differential effects on human T and NK cells. Eur J Immunol 2000; **30:** 1364–74.

41 Joshi PC, Zhou X, Cuchens M, Jones Q. Prostaglandin E_2 suppressed IL-15-mediated human NK cell function through down-regulation of common γ-chain. J Immunol 2001; **166:**885–91.

42 Murphy KM, Ouyang W, Farrar JD, Yang J, Ranganath S, Asnagli H, Afkarian M, Murphy TL. Signaling and transcription in T helper development. Annu Rev Immunol 2000; **18:**451–94.

43 D'Acquisto F, Sautebin L, Iuvone T, Di Rosa M, Carnuccio R. Prostaglandins prevent inducible nitric oxide synthase protein expression by inhibiting nuclear factor-κB activation in J774 macrophages. FEBS Lett 1998; **440:**76–80.

44 Kolenko V, Rayman P, Roy B *et al.* Downregulation of JAK3 protein levels in T lymphocytes by prostaglandin E_2 and other cyclic adenosine monophosphate-elevating agents: impact on interleukin-2 receptor signaling pathway. Blood 1999; **93:**2308–18.

45 Boer A-K, Drayer L, Vellenga E. Prostaglandin-E_2 enhances EPO-mediated STAT5 transcriptional activity by serine phosphorylation of CREB. Blood 2002; **100:**467–73.

46 Penix LA, Sweetser MT, Weaver WM, Hoeffler JP, Kerppola TK, Wilson CB. The proximal regulatory element of the interferon-γ promoter mediates selective expression in T cells. J Biol Chem 1996; **271:**31964–72.

47 Zhang F, Wang D, Boothby M, Penix L, Flavell RA, Aune TM. Regulation of the activity of IFN-γ promoter elements during Th cell differentiation. J Immunol 1998; **161:**6105–12.